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Neobulgaria alba sp. nov. and its *Phialophora*-like anamorph in native forests and kiwifruit orchards in New Zealand

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Abstract – Surveys of fungi associated with stained vascular tissue in kiwifruit vines in New Zealand have consistently revealed *Phialophora*-like fungi. Phylogenetic analyses based on DNA sequences have shown most of these to be *Leotiomycetes*. The teleomorph of the most common species isolated from stained kiwifruit wood has been found on fallen wood in native forests, and it is described here as *Neobulgaria alba* sp. nov. Other species isolated from kiwifruit wood matched *Cadophora* and *Mollisia* spp. reported from similar symptoms from kiwifruit and other hosts in other countries.

Key words – vascular staining, pathogen, *Actinidia deliciosa*, phylogeny, *Phaeoacremonium*

Introduction

Discoloured and decayed wood is common in the trunks of kiwifruit (*Actinidia deliciosa*) vines more than about 10 years old. The stained wood symptom is similar to that recorded in Italy where a number of fungi including *Phaeoacremonium* spp. and *Phialophora* spp. were isolated from diseased trunks (Di Marco et al. 2000). Surveys in New Zealand of fungi associated with these symptoms revealed several common *Phialophora*-like spp. (e.g. Manning et al. 2003, Manning & Currie 2007). Subsequent DNA sequencing showed them to represent *Leotiomycetes*, several matching described or known *Cadophora* spp. (unpubl. data).

Gams (2000) proposed using the genus *Cadophora* to accommodate some of the leotiomycete-related *Phialophora* spp. and Harrington & McNew (2003) clarified the taxonomy of these species. Based on ITS sequences, New Zealand isolates from kiwifruit wood match *Cadophora luteoolivacea* (e.g. Genbank accession HM116748), *Mollisia dextrinospora* (e.g. Genbank accession HM116746), and an apparently unnamed clade that includes "*Cadophora melinii*" sensu Prodi et al. (2008) but is genetically distinct from the ex holotype isolate of *C. melinii* (e.g. Genbank accession HM116752). This last species has been reported from wood and roots of various trees from Europe as Dark Septate Endophyte ITS Haplotype III in Grünig & Sieber (2005; Genbank accession number AY664502) and as *Phialophora malorum* aggregate in Lygis et al. (2005; Genbank accession number AY787725).

Phialophora-like anamorphs are spread throughout the *Leotiomycetes*. Examples discussed by Gams (1980) included representatives from the families *Helotiaceae*, *Dermateaceae*, *Hyaloscyphaceae*, and *Sclerotiniaceae*. Later authors have linked the *Phialophora*-like genera *Catenulifera* and *Phialocephala* to the leotiomycete genera *Hyphodiscus* (Hosoya 2002, Untereiner et al. 2006) and *Mollisia* (Grünig et al. 2009) respectively.

In this paper we report a *Phialophora*-like leotiomycete anamorph associated with a new *Neobulgaria* sp. The anamorph has been found in cultures grown from ascospores from apothecia collected in native forests and it is also commonly isolated into culture from diseased kiwifruit wood.

Methods

A survey of kiwifruit (*Actinidia deliciosa*) in 38 orchard blocks in South Auckland/Waikato (15), Bay of Plenty (15), Hawkes Bay (2), Nelson/Golden Bay (4), and Kerikeri (2) was carried out between 2002 and 2007. Samples of wood from symptomatic and non-symptomatic trunks were taken using a 4 mm diam core borer and small pieces of wood from these placed on Difco potato dextrose agar plates (PDA) with streptomycin and penicillin G added. The fungi isolated were grouped on the basis of cultural appearance and micro-morphology. Representative isolates were stored as a working collection in 10% glycerol at −80°C and later placed in permanent storage in liquid nitrogen in the ICMP culture collection, Landcare Research, Auckland. DNA sequences were generated from these isolates following the methods below.

Apothecia were collected during a survey of wood rotting fungi in native forests (Paulus et al. 2006). While the collections were still fresh, ascospores were shot from living apothecia onto agar plates; germinating ascospores were transferred to new plates, and following adequate growth, cultures were placed in permanent storage in liquid nitrogen in the ICMP culture collection. Collections were dried and deposited in the New Zealand Fungal Herbarium (PDD). Macroscopic appearance was described from field notes and from dried herbarium material, and microscopic features described following rehydration of herbarium material in 3% KOH with Meltzer's reagent added.

Table 1. Collections used to generate DNA sequences for Fig. 2 in addition to those from Wang et al. (2006b), with Genbank accession numbers.

Species*	Voucher[a]	SSU, ITS, LSU rDNA	Notes
**Cadophora luteoolivacea*	ICMP 18096	HM116765, HM116748, HM116760	ICMP 17109, 18084, 18085, 18097, 18098, 18099 from *Vitis vinifera* wood and ICMP 18092 from *Actinidia deliciosa* wood have matching SSU, ITS, and LSU sequences
Hyphodiscus hymeniophilus	MUCL 40275	DQ227258, DQ227258, DQ227258	
Hyphodiscus hymeniophilus	CBS 529.87	—, GU727555, GU727555	
**Mollisia dextrinospora*	ICMP 18083	HM116762, HM116746, HM116757	ICMP 17107, 17108, 17110, 17111, 17112 from *Actinidia deliciosa* wood have matching ITS sequences (other genes not sampled)
**Neobulgaria alba*	ICMP 18072	HM116761, HM116745, HM116756	ICMP 17113, 17114, 17115, 18073, 18074, 18075, 18076, 18077, 18078, 18079, 18080, from *Actinidia deliciosa* wood have matching SSU, ITS, and LSU sequences
**Neobulgaria alba*	ICMP 18394, culture from Holotype	HM116781, HM116783, HM116782	ICMP 18395 from fallen wood in native forest has matching SSU, ITS, and LSU sequences
Neobulgaria pura	CUP 063609	DQ257364, DQ257366, DQ257365	
Phaeomollisia piceae	UAMH 10851	EU434866, EU434836, —	

* Indicates sequences generated as part of this study.

[a] CUP; Cornell Plant Pathology Herbarium, Cornell University, Ithaca, USA. CBS; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. ICMP; International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand. MUCL; Industrial Fungi & Yeasts Collection, Belgian Co-ordinated Collections of Micro-organisms, Belgium. UAMH; University of Alberta Microfungus Collection and Herbarium, Alberta, Canada.

Apothecia were sectioned at about 10 µm thickness using a freezing microtome, and sections were mounted in lactic acid.

DNA was extracted from mycelium from cultures grown from both apothecia and stained wood using REDExtract-N-Amp Plant PCR Kits (Sigma, USA), following manufacturer's instructions. ITS sequences were obtained following the methods of Johnston & Park (2005). Amplification primers for ITS were ITS1 and ITS4 (White et al. 1990); for small subunit ribosomal DNA were NS1 and NS6 (White et al. 1990), and

for the large subunit ribosomal DNA were LROR and LR5 (Bunyard et al. 1994, Vilgalys & Hester 1990). The sequences newly generated for this paper (Table 1) have been deposited in Genbank.

DNA sequences were aligned using Clustal X (Larkin et al. 2007), then checked and edited manually. A Bayesian tree was estimated in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) with gaps treated as missing data, using a partitioned model, where each portion of the rDNA was assigned a model selected using the AIC method in MrModelTest 2.3 (Nylander 2004). The model selected for the 18S and 28S rDNA was GTR+I+Γ and for the 5.8S rDNA was SYM+I+Γ. The data set was run with 2 chains for 10 million generations, trees sampled every 1000 generations with a burn-in of 10%. Bayesian posterior probabilities were obtained from 50% majority rule consensus trees. In addition to the species recorded from New Zealand, taxa for the analysis were selected from Wang et al. (2006b) to represent genetic diversity across the *Helotiales*, plus the *Phialophora*-like species cited in Untereiner et al. (2006) and Grünig et al. (2009).

Neobulgaria alba P.R. Johnst., D.C. Park & M.A. Manning, **sp. nov.** Fig. 1

mycobank MB 518281; GenBank HM116781, HM116782, HM116783.

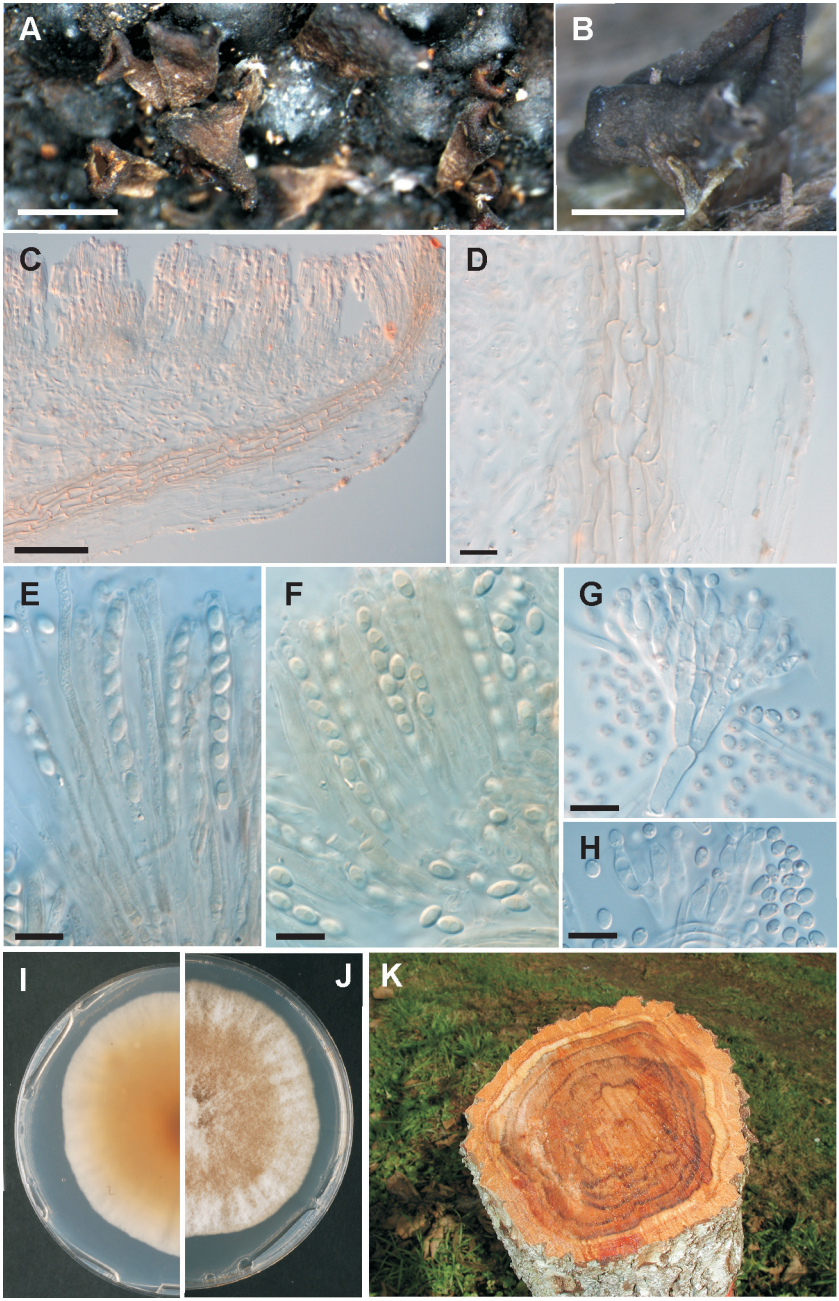
Ab Neobulgaria pura *ascsoporis (5–)5.5–6.5 × 3–3.5(–4) µm, apotheciis brunneis differens.*

Typification: New Zealand, vic. Ruatahuna, Tarapounamu, ridge towards Mangapae on western side of road, on fallen decorticated wood intermixed with *Rosellinia* ascomata, P.R. Johnston (D2022) & B.C. Paulus, 13 Dec 2006, (**Holotype**: PDD 91753; culture grown from holotype, ICMP 18394).

Etymology: *alba*, refers to the colour of the colonies on agar isolation plates, paler than most of the other fungi commonly isolated from discoloured vascular tissue.

Apothecia forming on decorticated wood; up to 3 mm diam., substipitate, dull, dark brown, glabrous; when dry receptacle dark brown, hymenium greyish-brown, stipe distinctively flattened. Ectal excipulum with 2 layers; outer layer 35–40 µm thick, comprising hyphae 1.5–2 µm diam. widely spaced in hyaline gel, oriented more or less parallel to receptacle surface; inner layer 30–35 µm thick comprising more or less parallel rows of broad-cylindric cells 5–7 µm diam. with walls thin, pale brown, nongelatinous. Medullary excipulum comprising a textura intricata of hyaline hyphae 2 µm diam. irregularly oriented and tangled within a hyaline gelatinous matrix. Subhymenium textura

Fig. 1. *Neobulgaria alba* (**A**, **C–F**, PDD 91753, holotype; **B**, PDD 91754; **G–J**, ICMP 19075). **A–B**. Apothecia (dry). **C**. Apothecium in vertical section. **D**. Apothecium in vertical section, detail of inner and outer ectal excipulum layers with hyphae regularly oriented, and medullary excipulum with hyphae irregularly oriented. **E**. Paraphyses and asci. **F**. Asci and ascospores. **G**. Conidiophores and conidiogenous cells from PDA cultures. **H**. Conidiogenous cells and conidia from PDA cultures. **I**. Bottom of 20-day-old colony on PDA. **J**. Top of 20-day-old colony on PDA. **K**. Stained kiwifruit wood from which *Neobulgaria alba* was isolated.
Scale bars; A–B = 1 mm; C = 50 µm; D–H = 10 µm.


A
B
C
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intricata, nongelatinous, comprising hyphae with pale brown cell contents. Paraphyses 2–2.5 µm diam., undifferentiated at apex, about same length as asci. Asci 70–110 × 5–6 µm, cylindric, tapering slightly to the subtruncate apex, wall thickened at apex, amyloid plug in the inner half of the wall, more intensely blue to the inside of the wall, 8–spored, uniseriate. Ascospores (5–)5.5–6.5 × 3–3.5(–4) µm, broad-elliptic, symmetrical in both planes, 0-septate, hyaline.

Anamorph in culture. Cultures on Difco PDA 60–70 mm after 20 days, aerial mycelium white, fine, cottony, quite sparse, pale brown agar surface visible through the mycelium. Culture pale yellow-brown in reverse, paler towards the more or less entire margin. Numerous small drops of more or less colourless conidial ooze scattered across surface of colony. Conidiophores penicillate, hyaline, cylindric basal cell 10–15 × 3–5 µm, with 3–4 levels of 2–3 times branching, cylindric cells arising from the basal cell, ending in a terminal conidiogenous cell. Conidiogenous cells 6–8 × 2.5–3.5 µm, more or less cylindric, tapering near apex, with single, apical conidiogenous locus, wall thickened at conidiogenous locus and with flaring collarette. Conidia hyaline, broadly ovate to subglobose, 4.5–5 × 3–3.5 µm.

ADDITIONAL SPECIMENS EXAMINED — New Zealand: Gisborne: vic. Ruatahuna, Te Waiiti, on fallen decorticated wood, P.R. Johnston (D2031) & B.C. Paulus, 4 Dec 2006, (PDD 91754, ICMP 18395). Buller: vic. Reeefton, Maimai Creek, on decorticated wood in running water, P.R. Johnston (D1377), 4 Oct 1998 (PDD 70861). Auckland: Waiuku, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM42), 21 Mar 2002 (ICMP 18072); Waiuku, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM43), 19 Mar 2002 (ICMP 18073); Waiuku, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM44), 15 Mar 2002 (ICMP 18074); South Auckland, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM48), 22 Mar 2002 (ICMP 18077); South Auckland, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM49), 22 Mar 2002 (ICMP 18077); Patamahoe, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM421), 22 Mar 2002 (ICMP 18077). Nelson, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM101), 8 Dec 2004 (ICMP 18080). Waikato, Pukekawa, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM46), 19 Mar 2002 (ICMP 18075); Pukekawa, on *Actinidia deliciosa* 'Hayward', swollen trunks of living vines, M.A. Manning (MM47), 19 Mar 2002 (ICMP 18076). Bay of Plenty: Te Puke, on *Actinidia deliciosa* 'Hayward' trunk decay, M.A. Manning (MM468), 13 Jul 2006 (ICMP 17114); Tauranga, on *Actinidia deliciosa* 'Hayward' trunk decay, M.A. Manning (MM493), 23 Mar 2007 (ICMP 17115); Te Puke, on *Actinidia deliciosa* 'Hayward' trunk decay, M.A. Manning (MM494), 20 Mar 2007 (ICMP 17113).

NOTES —Eleven species have been described in *Neobulgaria* (Index Fungorum 2010) — *N. caliciformis* Killerm., *N. faginea* (Peck) Raitv., *N. foliacea* (Bres.) Dennis, *N. lilacina* (Wulfen) Dennis, *N. margaritoidea* Killerm., *N. orientalis* Raitv. & Bogacheva, *N. parvata* V.P. Tewari & Ram N. Singh, *N. premnophila* Roll-Hansen & H. Roll-Hansen, *N. pura* (Pers.) Petr., *N. rupicola* V.P. Tewari

& Ram N. Singh, and *N. undata* (W.G. Sm.) Spooner & Y.J. Yao. The decision to describe *N. alba* as new is based in part on its genetic distinctness from collections putatively representing three north-temperate species, *N. pura* (DQ257364), *N. foliacea* (NPU45443), and *N. premnophila* (NPU45445), and in part on its geographic range. The 18S rDNA sequences of the three Northern Hemisphere collections differ from each other by 1–2 bases, whereas *N. alba* is 8 base pairs different.

Based on published descriptions, all of the described species differ from *N. alba* variously in ascospore size and septation, apothecial shape and size, and pigmentation in culture (Dennis 1956, 1971, Killerman 1929, Lizoň et al. 1998, Raitviir & Bogacheva 2007, Roll-Hansen & Roll-Hansen 1979, Seaver 1961, Tewari & Singh 1975). From the illustrations of Tewari & Singh (1975), *N. rupicola* appears to have the apothecial anatomy of *Ascocoryne*. From the description provided by Dennis (1954), based in part on material collected in tropical America, *N. alba* is very similar to *Ombrophila microspora* (Ellis & Everh.) Sacc. & P. Syd. However, Dennis (1954) described the asci of *O. microspora* as being barely thickened at the apex and as having an indistinct amyloid reaction. Lizoň et al. (1998) regarded *O. microspora* as a synonym of *N. pura*, a good illustration that species limits within the genus remain confused. Genetic studies on authentically identified specimens are needed to resolve the relationships between species of this genus, and of those that have been placed in *Ombrophila*.

Discussion

Common in living wood of mature kiwifruit vines but found also in native forests, the genetically distinct *Neobulgaria alba* is assumed to be a native New Zealand species that has moved from natural to human habitats. Data from Johnston (2010) show that many putatively native species of fungi have moved into modified habitats and formed associations with exotic host plants. Although represented by only three collections from native forests, this macroscopically insignificant fungus is assumed to be widespread in New Zealand forests. Its occurrence on kiwifruit suggests that it will have a naturally wide host range. Its biology in native forests is likely to be similar to that in kiwifruit orchards — as well as being apparently saprobic on fallen wood it will probably be found also in association with discoloured wood of living trees.

In New Zealand kiwifruit orchards *N. alba* has been found almost exclusively in association with swollen trunks and stained wood, with only one isolate from symptomless wood (Manning et al. 2003). Following inoculation of healthy wood of kiwifruit with *N. alba,* the fungus was subsequently re-isolated 8 months later from stained tissue surrounding the point of inoculation (unpublished data, M.A. Manning). Less commonly isolated from the same symptoms

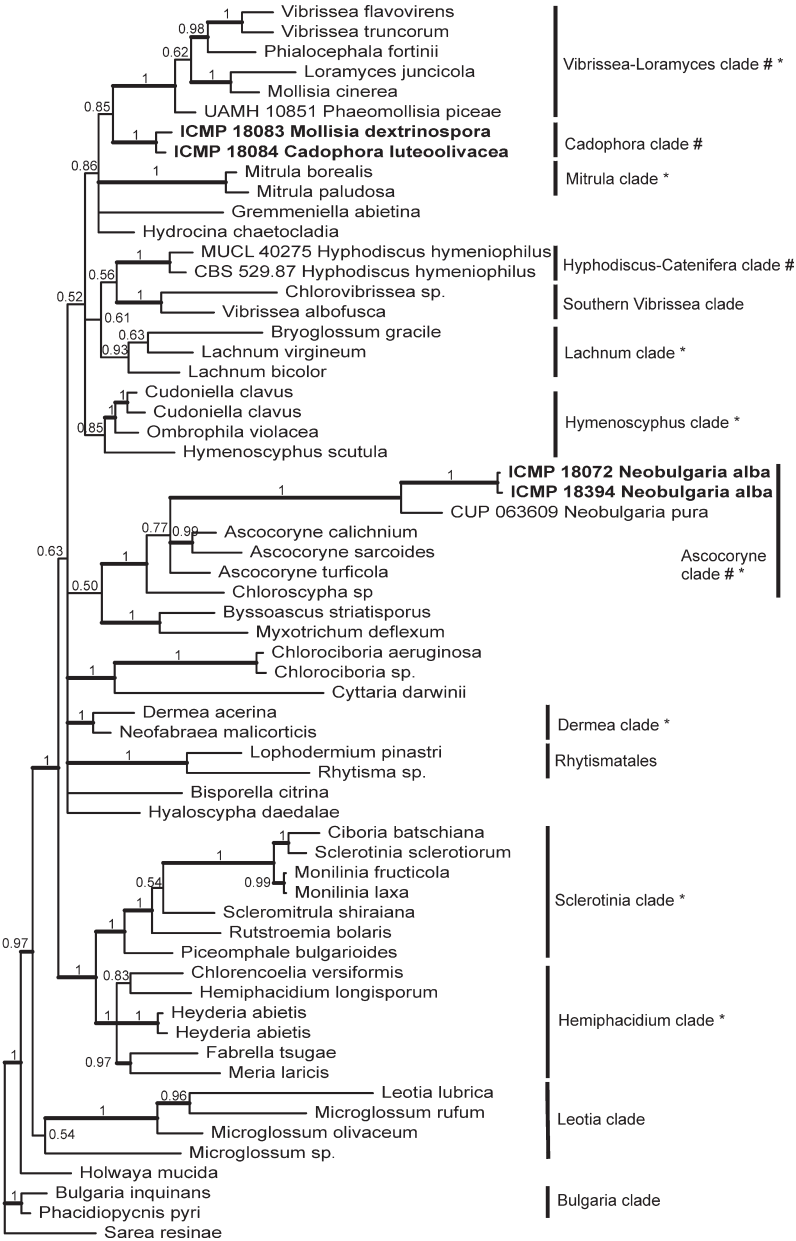
were putatively novel *Phaeoacremonium* spp. (e.g. ICMP 18093, Genbank HM116770, HM116776; ICMP 18094, Genbank HM116771, HM116777) and *Cadophora* spp. (Fig. 2 and Table 1). *Cadophora* and *Phaeoacremonium* spp. have been isolated from similarly discoloured wood in Italy, and pathogenicity tests again showed that they caused the symptoms (Di Marco et al. 2008). Field observations suggest that these fungi are unlikely to cause stem death, but they do impact on fruit yield. Fresh weight of fruit from New Zealand vines with disease symptoms averaged 12.6 g less than those from apparently healthy vines (Manning & Currie 2007).

The fungus from New Zealand kiwifruit has in the past been referred informally to "*Phialophora alba*" (e.g. Manning et al. 2003, Prodi et al. 2008). *Phialophora alba* was described from diseased wood by van Beyma (1943). He described a fungus with subglobose conidia, 3–4 × 2.7 µm, similar in size to *Neobulgaria alba*, but the ITS sequence from the type specimen (CBS 112.43, Genbank accession number HM116755) showed it to represent a *Paecilomyces* sp.

The genus *Neobulgaria* is characterised by the excipulum having a layer of non-gelatinous, cylindric cells sandwiched between gelatinous tissue to both the inside and outside. The outer gelatinous layer comprises narrow hyphae in a thick gel matrix, oriented more or less parallel to the receptacle surface, whereas the inner layer has hyphae irregular in orientation and less widely spaced in gel. Some species placed in *Ombrophila* have the same excipular structure (e.g. *O. microspora* as illustrated by Dennis 1954) and several authors have discussed the possibility that the two genera may be synonyms (e.g. Carpenter 1981, Dennis 1956, Verkley 1992). Baral & Krieglsteiner (1985) placed the genera in synonymy, recombining the type species of *Neobulgaria* in *Ombrophila*. However, the only genetic data available for the two genera indicate that they should be retained as distinct. *Ombrophila* belongs with some *Hymenoscyphus* spp. in what Wang et al. (2006a, b) suggested could represent a 'core' *Helotiaceae* sensu stricto clade, while *Neobulgaria* forms an unnamed clade with other genera with highly gelatinised excipular tissues, including *Ascocoryne* and *Chloroscypha*. Additional *Neobulgaria* sequences generated as part of this study support its position close to *Ascocoryne*.

Phylogenetic relationships of leotiomycete fungi with *Phialophora*-like anamorphs remain poorly resolved (Fig. 2). As suggested by Grünig et al.

Fig. 2. 50% majority-rule consensus phylogenetic tree based on Bayesian analysis of 18S rDNA, 5.8S rDNA, and 28S rDNA regions. Details of taxa with voucher numbers before the names are provided in Table 1, all other taxa are from Wang et al. (2006b). Bayesian posterior probabilities are shown above the edges, and those greater than 95% are indicated with bold lines. Informal clade names marked with * follow Wang et al. (2006a) and those marked with # are clades containing wood-staining fungi discussed in this paper. *Sarea resinae*, basal to the monophyletic *Leotiomycetes* in Wang et al. (2006b) was selected as the outgroup.


Vibrissea flavovirens
Vibrissea truncorum
Phialocephala fortinii
Loramyces juncicola
Mollisia cinerea
UAMH 10851 Phaeomollisia piceae
Vibrissea-Loramyces clade # *
ICMP 18083 Mollisia dextrinospora
ICMP 18084 Cadophora luteoolivacea
Cadophora clade #
Mitrula borealis
Mitrula paludosa
Gremmeniella abietina
Hydrocina chaetocladia
Mitrula clade *
MUCL 40275 Hyphodiscus hymeniophilus
CBS 529.87 Hyphodiscus hymeniophilus
Hyphodiscus-Catenifera clade #
Chlorovibrissea sp.
Vibrissea albofusca
Southern Vibrissea clade
Bryoglossum gracile
Lachnum virgineum
Lachnum bicolor
Lachnum clade *
Cudoniella clavus
Cudoniella clavus
Ombrophila violacea
Hymenoscyphus scutula
Hymenoscyphus clade *
ICMP 18072 Neobulgaria alba
ICMP 18394 Neobulgaria alba
CUP 063609 Neobulgaria pura
Ascocoryne calichnium
Ascocoryne sarcoides
Ascocoryne turficola
Chloroscypha sp
Byssoascus striatisporus
Myxotrichum deflexum
Ascocoryne clade # *
Chlorociboria aeruginosa
Chlorociboria sp.
Cyttaria darwinii
Dermea acerina
Neofabraea malicorticis
Dermea clade *
Lophodermium pinastri
Rhytisma sp.
Rhytismatales
Bisporella citrina
Hyaloscypha daedalae
Ciboria batschiana
Sclerotinia sclerotiorum
Monilinia fructicola
Monilinia laxa
Scleromitrula shiraiana
Rutstroemia bolaris
Piceomphale bulgarioides
Sclerotinia clade *
Chlorencoelia versiformis
Hemiphacidium longisporum
Heyderia abietis
Heyderia abietis
Fabrella tsugae
Meria laricis
Hemiphacidium clade *
Leotia lubrica
Microglossum rufum
Microglossum olivaceum
Microglossum sp.
Leotia clade
Holwaya mucida
Bulgaria inquinans
Phacidiopycnis pyri
Bulgaria clade
Sarea resinae

(2009), *Phialocephala*, *Acephala,* and their newly described genus *Phaeomollisia*, belong in the *Vibrissea-Loramyces* clade of Wang et al. (2006a). In our analyses, *Cadophora* forms a poorly supported sister relationship with the *Vibrissea-Loramyces* clade, and the position of *Hyphodiscus* remains unresolved amongst a group of the Wang et al. (2006a) clades including the *Vibrissea-Loramyces*, *Mitrula*, *Hymenoscyphus*, and *Lachnum* clades (Fig. 2).

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